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14. ABSTRACT
This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. The specific aims are to generate functional chromaffin-like cells from mesenchymal stem cells (MSCs) and to investigate the analgesic and anti-tolerance effects and the safety of chromaffin-like cells in animal models. We have conducted the proposed experiments as outlined in SOW. Specifically, we have produced chromaffin-like cells by reprogramming human MSCs (hMSCs) with the extracts of porcine adrenal chromaffin cells. We have harvested bone marrow tissues from rats and isolated, cultured, and expanded rat MSCs (rMSCs) for the targeted reprogramming by using cellular extracts of porcine adrenal chromaffin cells to produce chromaffin-like cells. Recently, we found that even naïve MSCs at their early passages (<passage 5) had analgesic effects in animals and had profound inhibitory effects on the development of morphine tolerance in a SH-SY5Y cellular model. Our research has led to one manuscript in press, one poster presentation, and one abstract in this first year of a 3 year project.

15. SUBJECT TERMS- Mesenchymal stem cells, Cell reprogramming, Pain management, Tolerance, Drug abuse, Cell cultures, Spinal transplantation of autologous stem cells, Animal behavioral tests

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INTRODUCTION:

Problems: Pain is a leading cause of disability among active duty and retired military personnel. Ineffective treatment often leads to pain-related impairments and drug abuse with long-term costs to both the military health and disability systems. Clinical trials have demonstrated that transplantation of allogeneic adrenal medullary tissue provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1, 2, 3, 4). There is often long-term pain relief without analgesic tolerance (one year in humans) (5). Following adrenal chromaffin cell transplantation, application of exogenous opiates further alleviated pain without dosage escalation (4); i.e., there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that transplantation of adrenal chromaffin cells ameliorated the problem of opioid tolerance (2, 6). Transplanted adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, gamma aminobutyric acid, indolalkylamines, and other neuropeptides (7, 8). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9, 10, 11), suggesting that these effects are mediated largely by opioids and catecholamines released by these cells. The anti-tolerance analgesic effects produced by the transplantation of adrenal chromaffin cells may be attributed to a synergistic action of endogenous analgesic molecules released by the transplanted cells.

However, clinical practice has been hindered due to the limited availability of suitable human adrenal tissue, genetically well-matched donors in particular, to serve as grafts. Mature chromaffin cells are post-mitotic when they produce enkephalines and catecholamines. The expansion in culture of these cells is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in a thermal pain model of rat and non-human primate (12, 13, 14, 15), with a gradual decline in analgesic efficacy that can be prolonged by administration of immunosuppression (13, 15), suggesting that xenogeneic chromaffin cells elicit host immunological rejection to the transplants, and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of the transplants. In addition, there has been concern regarding pathogen contamination of these xenogeneic materials, such as bovine spongiform encephalopathy for bovine chromaffin cell transplantation. Thus, the ideal cell source would be the autologous chromaffin cells derived from the patient's own tissue.

Emerging cell reprogramming technology allows production of chromaffin-like cells (CLCs) from autologous stem cells (16, 17, 18, 19), which can be generated epigenetically to secrete analgesic substances, anti-inflammatory factors, and immunological modulating molecules, and used for the management of chronic pain and prevention of drug abuse. In our preliminary studies, we have successfully generated functional chromaffin-like cells by reprogramming human mesenchymal stem cells (hMSCs) with the cellular extracts of porcine chromaffin cells (20, 21, 22). We hypothesized that transplantation of these reprogrammed CLCs, autologous CLCs in particular, will produce significant long term analgesic and anti-tolerance effects without any major adverse effects and immunological rejection. This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. They will work jointly on the Specific Aims to generate CLCs from hMSCs and rat MSCs (rMSCs) and to investigate the analgesic and anti-tolerance effects and the safety of CLCs in animal models. This project is anticipated to establish an innovative therapy that will have a profound impact on pain management and drug abuse prevention, two of the major barriers facing the military and society at large. Such a therapy will fundamentally reduce the need for exogenous opioid medications and minimize the risk of prescription drug abuse and addiction. It can be used in hundreds of thousands of patients with a wide range of cancer- and non-cancer pain states to improve quality of life and save billions of dollars for the military health and disability systems.

BODY:

This partnering PI option project has been progressing as planned. We here report our research progress and accomplishments in the first 12 months of this 36 month project as outlined in our original statement of work (SOW) for the partnering PI's specific tasks in the Qu lab in details including methodology, results, and discussion of data.

Task 1 (month 1-32): Generate functional chromaffin-like cells: i.e., use porcine chromaffin cell nuclear and cytoplasmic extracts to guide hMSCs and rMSCs to develop into chromaffin-like cells (CLCs) through reprogramming technology. Task 1 is in progress.

We have produced human CLCs (hCLCs) by reprogramming hMSCs with the extracts of porcine adrenal chromaffin cells based on our established protocol (20, 21, 22). For details, see "Targeted cell reprogramming produces analgesic chromaffin-like cells from human mesenchymal stem cells" (ref. 27, PMID:23394594), which has been published in the journal of Cell Transplantat and attached to the report as an appendix. The experiments for the production of rat CLCs from rMSCs (rCLCs) is on-going in the lab. Task 1 is in progress.

1a. Acquisition of porcine chromaffin cells for CLC production (months 1-6):

Task 1a is in progress.

We have obtained porcine adrenal glands (x10) from a Chicago slaughterhouse and purchased porcine adrenal glands (x17) from Sierra For Medical Science (Whittier, CA), respectively. The adrenal chromaffin cells have been successfully isolated from these porcine adrenal glands, purified, and cultured. Isolation of adrenal chromaffin cells was carried out using a method we have reported previously (20, 21, 22). Freshly isolated chromaffin cells were suspended and plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) containing Dulbecco's modified eagle medium/F12 (DMEM/F12, 1:1; Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma, St Louis, MO) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA) fed by replacing culture media twice per week and used for experiments within four weeks after culturing because mature chromaffin cells are post-mitotic and do not survive longer in cultured conditions.

1b. Cell reprogramming (months 4-32):

Task 1b is in progress.

hMSC culture: hMSCs were purchased from Cambrex (Walkersville, MD) and AllCells (Emeryville, CA), cultured, and expanded using the protocol previously developed in our laboratory (20, 21, 22). In brief, hMSCs were plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) at a concentration of $1x10^5$ cells/cm^2 and cultured in 20 ml growth medium consisting of DMEM (Gibco, Grand Island, NY), an antibiotic-antimycotic mixture (1:100, Invitrogen, Carlsbad, CA), and FBS (Stem Cell Technologies, Vancouver, BC, Canada), incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA), fed by replacing half of the culture media twice a week, and passaged regularly after reaching about 80% confluency. hMSCs that underwent less than 10 passages were used for the experiments.

rMSC culture: Bone marrow tissues derived from adult male Sprague-Dawley (SD) rats (x22) were obtained from Dr. Cheng's lab. Mononuclear bone marrow cells were isolated and incubated at 37°C in a 5% CO2 humidified incubator. Homogeneous rMSCs were isolated based on their adherence to plastic in culture and expanded. The conditions for culturing and expanding rMSCs as well as cell passaging were similar to those used for hMSCs.

Reprogramming MSCs with the extracts of porcine chromaffin cells: The cell reprogramming processes for producing hCLCs and rCLCs are similar.

a. Preparation of porcine chromaffin cell extracts: Cultured porcine chromaffin cells were counted and washed in PBS and in cell lysis buffer (20mM HEPES, pH 8.2, 50 mMNaCl, 5mM $MgCl_2$, 1mM dithiothreitol, and protease inhibitors) (Sigma, St Louis, MO), sedimented at 400×*g*, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200µl aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed, and confirmed by microscopic observation. The lysate was centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use.

b. Permeabilization of MSCs with SLO: MSCs were suspended, washed in Ca^{2+}- and Mg^{2+}-free PBS, and centrifuged at 120×*g* for 5 min at 4°C. The collected MSCs were resuspended in aliquots of $1x10^5$ MSCs/100µl of Ca^{2+}- and Mg^{2+}-free PBS in 1.5 ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich, St Louis, MO) at a final concentration of 200ng/ml and incubated in an H_2O bath at 37°C for 50 min with occasional agitation. The cell samples were then placed on ice, diluted with 200µl cold PBS, and sedimented at 150×*g* for 5 min at 4°C.

c. Reprogramming MSCs with the extracts of porcine chromaffin cells: The permeabilized MSCs ($1x10^5$) were resuspended in 100µl of the extracts of porcine chromaffin cells in a 1.5ml tube containing an ATP regenerating system (1mM ATP, 100µM GTP, and 1mM of each NTP, 10mM creatine phosphate, 25µg/ml creatine kinase) (Sigma, St Louis, MO). Cell samples were incubated in an H_2O bath at 37°C for 1 hr with occasional agitation. To reseal the membranes of MSCs, 1ml of DMEM containing 2mM $CaCl_2$ and antibiotics were added to the tube and incubated at 37°C for an additional 1 hr. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS and reprogrammed MSCs were transferred to culture flasks at a concentration of $1x10^5$ cells/cm^2 and expanded continuously for subsequent experiments.

1c. Characterization of CLCs (months 4-12):

Task 1c is in progress.

hCLC expression of key genes (RT-PCR) and molecular markers of chromaffin cells (immunocytochemistry), cell proliferation (BrdU immunocytochemistry), and cell viability (trypan blue) were performed (27). rCLC expression of key genes (RT-PCR) and molecular markers of chromaffin cells (immunocytochemistry), cell proliferation (BrdU immunocytochemistry), cell viability (trypan blue), cell karyotypes (Giemsa staining), and cell apoptosis (TUNEL) are on-going experiments conducted in the lab.

Gene expression of hCLCs: One week after cell reprogramming with the cellular extracts of porcine chromaffin cells, RT-PCR was performed to examine the expression of hPPE. The molecular size of the RT-PCR product for the human preproenkephalin (hPPE) gene, a precursor for enkephalin opioid peptides was 425bp. As expected, naïve hMSCs demonstrated a low level of inherent hPPE gene expression. The chromaffin-like cells generated from the reprogrammed hMSCs showed a significantly

enhanced expression profile for gene hPPE compared to that of naïve hMSCs (P<0.01, Figure 1 (p26) and Figure Legend (p19) in the publication of the Appendices), suggesting that cell reprogramming further increases the expression of hPPE genes in the population of reprogrammed hMSCs.

Immunocytochemical examination of hCLCs: Following cell reprogramming, morphological changes in hMSCs were observed within the first few days; i.e., reprogrammed hMSCs became smaller and rounder. Five days later, the cells reverted to fibroblast-like shapes. The expansion of these cells in culture was at a slightly slower speed (doubling time: about 84 hours) in the first week and recovered at a normal dividing rate (doubling time: about 72 hours) similar to that of naïve hMeSCs. Two weeks after cell reprogramming, immunocytochemical examination showed that most of the resultant chromaffin-like cells (≥90%) expressed a strong immunoreactivity for Met-enkephalin and TH, specific cytoplasmic markers for adrenal chromaffin cells (Figure 3 (p27) and Figure Legend (p19-20) in the publication of the Appendices). Interestingly, BrdU-positive staining was detected in a subpopulation of chromaffin-like cells, suggesting that some chromaffin-like cells may have retained a similar proliferative capability as that of hMSCs and could be expandable in cell cultures.

1d. The phenotypic stability and secretory function CLCs (month 12-24):

Task 1d is in progress.

Phenotypic stability of hCLCs: These hCLCs remained stable phenotypes and expanded in cultures with an average cell doubling time of about 72 hours up to one month, undergoing about 8 passages. We did not maintain the culture of the cells post the one month time point after cell reprogramming. The secretory function hCLCs were investigated and reported as follows. The experiments on phenotypic stability and secretory function for rCLCs will be initiated soon.

Met-enkephalin secretion of hCLCs: In parallel to the time point examined for the expression of hPPE genes by RT-PCR (one week post-cell reprogramming), the serum-supplemented culture medium for hCLCsand naïve hMSC cultures was replaced by a serum-free culture medium. Twenty-four hours later, the medium was collected and purified for Met-enkephalin detection by immunoblot assays. The level of Met-enkephalin released by hCLCswas significantly augmented compared to that released by naïve hMSCs for the same number of cells ($1x10^5$ cells/well) in serum-free cultures (P<0.01, Figure 2 (p26) and Figure Legend (p19) in the publication of the Appendices). Although naïve hMSCs were able to produce and release a low level of Met-enkephalin into the serum-free medium, augmented production and secretion of Met-enkephalin opioid peptides in hCLCs were consistently observed in each of the four independent experiments.

As mentioned above, we have generated functional chromaffin-like cells by reprogramming hMSCs with the cellular extracts of porcine chromaffin cells. These chromaffin-like cells generated from the reprogrammed hMSCs displayed a significant increase in expression of human preproenkephalin (hPPE), a precursor for enkephalin opioid peptides, compared to the inherent expression of hPPE in naïve hMSCs. The resultant chromaffin-like cells not only expressed the key molecular markers of adrenal chromaffin cells, such as tyrosine hydroxylase (TH) and methionine enkephalin (Met-enkephalin), but also secreted opioid peptide Met-enkephalin in cultures. Intrathecal injection of these chromaffin-like cells in rats produce significant antinociceptive effect *in vivo* for responses to both Aδand C thermal stimuli (Figure 4 (p27) and Figure Legend (p20) in the publication of the Appendices), which is at similar efficacy observed with porcine chromaffin cell transplantation without using immunosuppressants (12). Our results suggest that analgesic chromaffin-like cells can be produced from an individual's own tissue-derived stem cells by targeted cell reprogramming for chronic pain management. Transplantation of cells derived from individuals' autologous tissues into the same individuals would be safe and

immunocompatible compared to xeno- and allo-transplants. In practice, hMSCs can be derived from a patient's own tissues and could not only be prepared to become isogeneic through this cell reprogramming approach, but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous chromaffin-like cells could be the most desirable alternative to adrenal chromaffin cells for potential therapeutic purposes. Robust and long-lasting analgesic effects of autologous chromaffin-like cells are expected because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the transplanted cells. These research findings have been presented at the Military Health System Research Symposium (MHSRS), Harbor Beach Marriott, Ft Lauderdale, Florida, August 13-16, 2012, and have been published online in the journal of Cell Transplant (see the attached Poster and Publication in the Appendices). Support of DOD grant is acknowledged in the presentation and publication.

Task 2 (month 4-36): Determine the analgesic and anti-tolerance effects and the safety of chromaffin-like cells in neuropathic pain rats: The animal surgery, behavioral tests, and biochemistry will be performed in Dr. Cheng (Initiating PI) lab, Cleveland Clinic. The molecular biology and histology experiments will be done in Dr. Qu (Partnering PI) lab, UIC.

Task 2 is in progress. As assigned in the Task 1 for Partnering PI, hMSCs and the generated hCLCs have been shipped to Dr. Cheng's lab where a transplantation study was conducted to investigate their analgesic effect in a rat model of neuopatic pain and promising preliminary data was generated. rMSCs have been successfully isolated from the bone marrows sent by the Cheng lab, cultured, and expanded in the Qu lab. While parts of the naïve rMSCs have been shipped to the Cheng lab for ongoing *in vivo* experiments of cell transplantation in rats, parts of the rMSC cultures were maintained in the Qu lab for producing rCLCs. The experiments for generating rCLCs are on-going and the generated rCLCs will be shipped to the Cheng lab for autologous CLC transplantation studies in the same rats with neuopathic pain.

2a. (months 4-30): Generate neuropathic pain model and perform transplantation experiments (72 rats); Perform analgesic evaluation (foot withdrawal thresholds to von Frey test and the Hargreaves test); CSF sampling and measuring concentrations of enkephalins (ELISA) and catecholamines (HPLC).

Task 2a is in progress. The neuropathic pain models have been successfully established in the Cheng lab according the methodology we described previously (23, 24, 25). The research progress and accomplishments for the initiating PI's tasks as outlined in the approved SOW have been reported separately by Dr. Cheng.

2b. (months 18-24): Determine cell dose response curve (60 rats) (foot withdrawal threshold tests and concentration of enkephalins and catecholamines in the CSF).

Task 2b will be initiated soon in the initiating PI Dr. Cheng's lab.

2c. (months 25-36): Determine anti-tolerance effects of autologous CLCs to repeated morphine injections (24 rats) (foot withdrawal thresholds to thermal and mechanical stimuli).

The experiments of anti-tolerance effects of autologous CLCs in animals listed in Task 2c will be performed in the initiating PI's lab in the 3rd year of the project. Previous studies demonstrated that transplantation of real adrenal chromaffin cells play an important role in the inhibition of opioid tolerance, which may be attributed to a synergistic action of endogenous molecules released by these cells. We anticipated that MSCs-derived CLCs may also produce antitolerance effects to repeated morphine uses. In

order to provide reliable *in vitro* data for animal experiments as listed in Task2c, we recently examined the anti-tolerance effects and the possible mechanisms of cell therapies by a co-culture system of naïve hMSCs and SH-SY5Y cellsdifferentiated neurons and found that hMSCs at the early passages (<passage 5) showed significant inhibitory effects on the morphine-induced tolerance in SH-SY5Y differentiated neuronal cells. experiment for investigating the anti-tolerance effects of CLCs will be followed. The results have been presented at the 3rd Annual Research Forum-Extravaganza 2012, Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, September 19, 2012 (26), and a manuscript is in preparation. Support of DOD grant is acknowledged in the presentation.

2d. (months 6-36): Determine the safety of chromaffin-like cell therapy: Determine the fate of the transplanted cells and their phenotypic stability (immunocytochemistry for specific cell markers, Qu lab); Determine host responses to graft (immunological and histological staining, Qu lab).

Task 2d is in progress.

Based on the current data, the animals with hCLC, hMSC, or rMSC transplantation maintained normal food and water intake, locomotor function, and body weight. The immunological and histological examination of the transplants will be initiated upon receiving the postmortem samples.

Task 3 (months 25-36): Determine the safety of chromaffin-like cells in SCID mice:

3a. (months 25-26): Transplantation of rat CLCs in SCID mice.
3b. (months 25-32): Animal general health observation including tumor formation.
3c. (months 33-36): postmortem histological examination to rule out tumor formation in the spinal cord and brain, as well as other major organs outside the CNS.

The experiments listed in Task 3 are not initiated yet.

KEY RESEARCH ACCOMPLISHMENTS:

- A new finding that naïve hMSCs at the early passages (<passage 5) demonstrated inhibitory effects on the development of morphine tolerance in SH-SY5Y cellular model;

- Naïve MSCs at the early passages (<passage 5) may have moderate analgesic effects in animal model;

- A manuscript "Targeted cell reprogramming produces analgesic chromaffin-like cells from human mesenchymal stem cells“ has been published in the journal Cell Transplantation. Support of DOD grant is acknowledged in the publication;

- One poster presented at Military Health System Research Symposium (MHSRS) in 2012. Support of DOD grant is acknowledged in the presentation;

- One poster was presented at the 3rd Annual Research Forum-Extravaganza, Department of Psychiatry, University of Illinois at Chicago, 2012. Support of DOD grant is acknowledged in the presentation

REPORTABLE OUTCOMES

Posters:

Sun JH, Yang HN, and **Qu T*,** Human mesenchymal stem cells from bone marrow attenuate morphine tolerance *in vitro,* The 3rd Annual Research Forum-Extravaganza, Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, September 19, 2012

Shi GB, Ma K, Pappas GD., and **Qu T*,** Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells, Military Health System Research Symposium (MHSRS), Harbor Beach Marriott, Ft Lauderdale, Florida, August 13-16, 2012

Publication:

Qu T*, Shi GB, Ma K, Yang H, Duan WM, and Pappas GD., Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells, Cell Transplant., 2013, PMID:23394594

Manuscript in Preparation:

Sun JH, Yang HN, and **Qu T*,** Human mesenchymal stem cells attenuate morphine tolerance 2012 (In preparation)

CONCLUSION:

Our research is progressing well as planned in the approved SOW and will have a substantive impact on the final goals of this DOD grant. In addition, we are excited about the recent discovery that even naïve MSCs in the early passages (<passage 5) without cell reprogramming may lead to inhibition of pain and morphine tolerance. Our confidence is further supported by the recent findings from other independent investigators that MSCs not only can reduce pain but also inhibit activation of microglia and astrocytes which play a critical role in the development and maintenance of chronic pain (28, 29). We are continuing to implement our research plans as outlined in our original research proposal by focusing to the approved SOW. We will compare the therapeutic value, analgesic effect, and anti-tolerance potentials of the early passaged naïve MSCs and the CLCs (reprogrammed MSCs) to expand our research and determine the best cell types for clinical application. We will also compare intravenous vs. intrathecal application of MSCs to determine the best route of clinical use. In this way, we hope to move much closer to be able to treat chronic pain with this novel treatment to produce long-lasting analgesic and anti-tolerace effects.

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APPENDICES:

- 2 posters
- 1 publication

UNIVERSITY OF ILLINOIS AT CHICAGO

Department of Psychiatry

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POSTER TITLE	Human mesenchymal stem cells attenuate morphine tolerance
DISEASE/KEY WORDS:	hMSCs, morphine, tolerance, cAMP, fluorescent immunoassy
AUTHORS:	Jinhua Sun, Hongna Yang, Tingyu Qu*
MENTEE CATEGORY:	Post-Doctoral Associate **RESEARCH MENTOR:** Tingyu Qu*
BACKGROUND:	Human mesenchymal stem cells (hMSCs) affect the inflammatory milieu and release endogenous analgesic molecules such as met-enkephalins. These findings have led researchers to consider hMSCs as a treatment for various diseased conditions including painful states. Morphine is one of the effective pain killers commonly used in today's clinics. We recently investigated whether hMSCs can attenuate the morphine tolerance in morphine pre-treated SH-SY5Y cells by a cell co-culture system.
METHODS:	SH-SY5Y cell were pre-treated by 10μM morphine for 24h and then co-cultured with different passages of hMSCs or their conditioned media for 36 hours. cAMP level, which is involved in opioid tolerance development as one of the cellular mechanisms, was examined in the tolerated SH-SY5Y cells with and without cell co-cultures by a fluorescent immunoassay.
RESULTS:	As expected, chronic morphine treatment produced an up-regulated level of cAMP in SH-SY5Y cells. hMSCs at early passage (≤passage 5) significantly inhibited the up-regulation of cAMP level in SH-SY5Y cells at the ratio of 1:5 and 1:25 (hMSCs/SH-SY5Y cells), with a gradual decline when the ratio of MSCs/SH-SY5Y cells was further decreased in co-cultures. However, hMSCs at late passage (≥passage 10) and conditioned medium of hMSCs show no significant inhibitory effects on the up-regulation of cAMP level in SH-SY5Y cells, suggesting that the effects of hMSCs in attenuating morphine tolerance seem to be cell-cell contact and cell passage dependent.
CONCLUSIONS:	These results provide new information that hMSCs may attenuate the development of morphine tolerance produced by morphine application in the management of chronic pain.

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Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells

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INTRODUCTION

Transplantation of allogeneic adrenal chromaffin tissues, which release endogenous opioid peptides and catecholamines, produces significant analgesic effects in patients with terminal cancer pain. After spinal transplantation, these patients showed increased levels of endogenous opioids and catecholamines in the cerebrospinal fluid with robust and long-lasting pain alleviation accompanied by an inhibition of the development of tolerance to exogenous opioid analgesics. The anti-tolerance effects produced by the transplantation of adrenal chromaffin cells may be attributed to a synergistic action of endogenous analgesic molecules released by the transplanted cells.

Although transplantation of adrenal chromaffin cells demonstrated promise of favorable outcomes for pain relief in patients, there is a very limited availability of suitable human adrenal tissues to serve as grafts. Alternative xenogeneic materials, such as porcine and bovine adrenal chromaffin cells, present problems of immune rejection and possible pathogenic contamination. To overcome these problems, we tested an approach to reprogram human bone marrow-derived mesenchymal stem cells (hMeSCs) using the cellular extracts of porcine chromaffin cells and produced a new type of cells, chromaffin-like cells. These newly-generated chromaffin-like cells acquired some key functional characteristics of adrenal chromaffin cells such as synthesizing and secreting analgesic opioid peptides. Spinal transplantation of these chromaffin-like cells provided significant analgesic effects in an animals. Our ultimate goal is to establish an effective therapy for persistent chronic and intractable pain by utilization of chromaffin-like cells produced from autologous hMeSCs of individual patients. Success of the proposed studies will eventually reduce the need for exogenous opioid medications, with a potential to avoid tolerance as demonstrated in the clinical trials of adrenal chromaffin cell transplantation in humans.

MATERIAL AND METHODS

1)Production of chromaffin-like cells from hMeSCs:

Cell cultures: hMeSCs (Walkersville, MD) were cultured and expanded per the protocol developed in our laboratory (1, 2). Porcine (4 month old) adrenal glands were obtained from a local slaughterhouse and immediately placed in ice-cold Locke's buffer supplemented with antibiotics for processing. The isolation, purification, and culturing for porcine chromaffin cells were performed as described in our previous study (3).
Cell reprogramming: The experimental procedures for cell reprogramming are based on the method developed by Hakelien and Collas (4, 5). Cultured porcine chromaffin cells were washed in PBS and in cold cell lysis buffer (20 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors, Sigma-Aldrich), centrifuged at 400g, and resuspended in 1 volume of cell lysis buffer. Cells were then sonicated with a pulse sonicater in short pulses until all cells and nuclei are lysed. The lysate were centrifuged at 15,000g for 15 min at 4°C and the supernatant were aliquoted and stored in liquid nitrogen for later use. Before applying to cell reprogramming, the protein concentration of the cells extracts was determined. To effect cell reprogramming, hMeSCs were reversibly permeabilized with the cholesterol-binding toxin streptolysin O (SLO, Sigma-Aldrich) and $1x10^5$ hMeSCs were incubated in 100µl nuclear and cytoplasmic extracts of $1x10^7$ porcine chromaffin cells containing an ATP-regenerating system (1mM ATP, 10 mM creatine phosphate, 25µg/ml cteatine kinase, 100µM GTP, and 1mM of each NTP) for 1hr at 37°C in a water bath. Following incubation, hMeSCs were resealed with $CaCl_2$ and cultured continuously for the following investigations.
RT-PCR: To characterize the expression of human preproenkephalin (hPPE), a precursor for enkephalin, in reprogrammed and naïve hMeSCs (unreprogrammed), total RNA from cell cultures was isolated at 2 weeks after cell reprogramming with TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with RNAase free DNAase (Promega, Madison, WI). The concentration of the RNA was quantified by absorbance at 260 nm. RT-PCR was performed using SuperScript One-Step RT-PCR approach (Invitrogen) using gene specific primer set for hPPE: 5'-ACATCAACTTCCTGGCTTGCGT-3' and 5'-GCTCACTTCTTCCTCATTATCA-3', with β-actin as an internal control. RT-PCR products were quantified using Qgel 1D program (Stratagene) and expressed as hPPE/β-actin ratio. Three independent experiments were performed for this study.
Immunoblot: The production and secretion of Met-enkephalin opioid peptide in cell cultures were examined using an immunoblot assay. In brief, reprogrammed hMeSCs were re-suspended from cell cuture flask and transferred into a 12 well culture plate containing serum-supplemented growth medium at a concentration of $1x10^5$/well, while naïve hMeSCs with the same number of cells ($1x10^5$/well) served as controls. After recovering overnight, the serum-supplemented growth medium for hMeSC cultures was replaced by serum-free medium (DMEM with antibiotic-antimycotic). Twenty-four hours later, medium was collected and purified with YM-30 microcon (Millipore Corp., Bedford, MA). Purified medium samples (200µl for each) obtained from reprogrammed or naïve hMeSCs were applied on Hybond ECL nitrocellurose membrane (Amersham Life Science, Piscataway, NJ) using the Slot Blot Hybridization Manifold (GENEMate, Kaysville, UT). The membranes were incubated with specific Met-enkephalin antibody made in rabbit (1:600, ImmunoStar Inc., Hudson, WI) followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3000, Amersham Biosciences). The membranes were then revealed with ECL Plus detection reagent and exposed to Hyperfilm ECL (Amersham Biosciences). The films were developed and scanned into a computer. Quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH). Data was expressed as mean ± SEM of 4 independent experiments.

2) Immunocytochemistry:

Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks post-cell reprogramming. Naïve hMSCs served as controls. Cell samples in eight-well culture chambers (Thermo Scientific, Swedesboro, NJ) were washed with PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma, St Louis, MO) and 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min, followed by incubation with mouse anti-TH (1:300; Sigma, St Louis, MO), or rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI) antibodies overnight at 4°C. Then, the cells were washed in PBS and incubated with their corresponding secondary antibodies, including FITC-conjugated donkey anti-mouse IgG or rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies for 2 hr at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA), and viewed by immunofluorescence microscopy (Zeiss, Jena, Germany).

3) Analgesic effects of chromaffin-like cells in rats:

Adult male Sprague Dawley (SD) rats (n=12, 250–300g) were used for this experiment. SD rats were lightly anesthetized with pentobarbital (35mg/kg, i.p.) and the baseline of foot withdrawal latencies were measured without cell transplantation using high (Aδ nociceptor: 6.5°C/second) and low (C nociceptor: 0.9°C/second) rates of radiant heating on the dorsal surface of the feet. The latencies from the onset of the stimulus to foot withdrawal responses were measured over 1 hour at 10 min intervals. After baseline for foot withdrawal responses was established, $1x10^5$ chromaffin-like cells generated from hMeSCs were transplanted into the rats (n=6) intrathecally under deep anesthesia with pentobarbital (50mg/kg, i.p.). Control rats (n=6) received injection of the same amounts of naïve hMeSCs. Rats were not immunosuppressed. Foot withdrawal latencies were re-measured at 1 week after cell transplantation and then weekly at one week intervals until the latencies returned to baseline. The foot withdrawal latencies were expressed as mean ± SEM. Student's t-test was used to compare the response latencies of chromaffin-like cell transplantation with baseline and that of naïve hMeSC transplantation.

RESULTS

1) Reprogrammed hMeSCs have a significantly enhanced expression for gene hPPE compared to that of naïve hMeSCs (Fig. 1, P<0.01) and a significant augmented release of Met-enkephalin opioid peptide in the serum-free culture medium compared to that of naïve hMeSCs for the same number of the cells (Fig. 2, P<0.01).
2) Immunocytochemical examination demonstrated that these reprogrammed hMeSCs not only expressed Met-enkephalin but also strongly express TH, an enzyme controlling the rate-limiting step of catecholamine biosynthesis, suggesting the potential of these cells to produce catecholamines. A strong immunoreactivity for specific markers of chromaffin cells, Met-enkephalin and TH, was observed in most of the reprogrammed hMeSCs (≥90%, Fig. 3).
3) Transplantation of chromaffin-like cells produced a significant analgesic effects in rats on both Aδ nociceptor- and C nociceptor-evoked responses and the analgesic effects lasted for about 3 weeks without immunosuppression (Fig. 4, P<0.05). Transplantation of naïve hMeSCs in rats showed response latencies similar to those of rats without cell transplantation in previous studies, with no detectable analgesic effects.

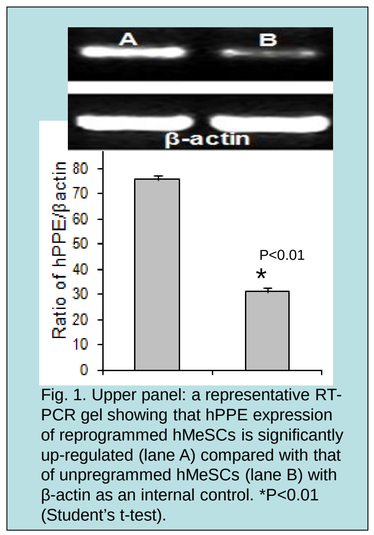

Fig. 1. Upper panel: a representative RT-PCR gel showing that hPPE expression of reprogrammed hMeSCs is significantly up-regulated (lane A) compared with that of unpregrammed hMeSCs (lane B) with β-actin as an internal control. *P<0.01 (Student's t-test).

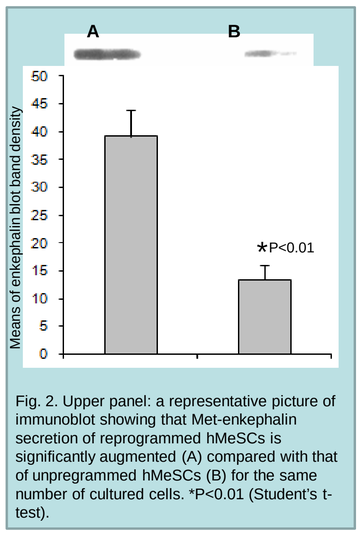

Fig. 2. Upper panel: a representative picture of immunoblot showing that Met-enkephalin secretion of reprogrammed hMeSCs is significantly augmented (A) compared with that of unpregrammed hMeSCs (B) for the same number of cultured cells. *P<0.01 (Student's t-test).

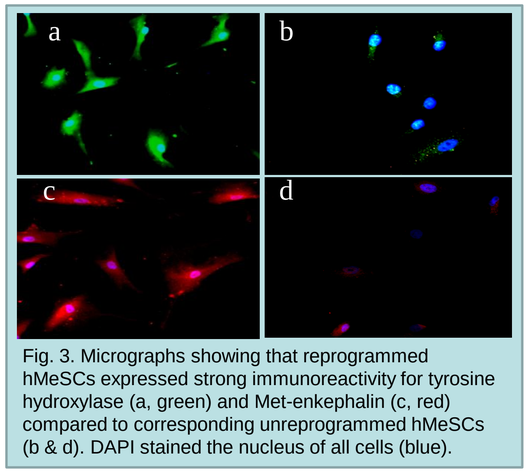

Fig. 3. Micrographs showing that reprogrammed hMeSCs expressed strong immunoreactivity for tyrosine hydroxylase (a, green) and Met-enkephalin (c, red) compared to corresponding unreprogrammed hMeSCs (b & d). DAPI stained the nucleus of all cells (blue).

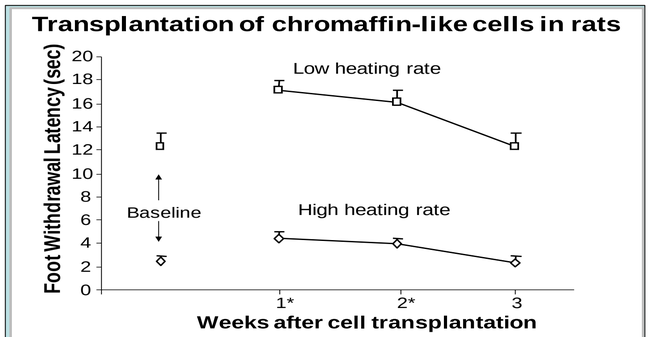

Figure 4. Transplantation of 100,000 chromaffin-like cells into the subarachnoid space increased foot withdrawal latencies evoked by both high (A-delta nociceptor) and low (C nociceptor) heating rates in rats for at least three weeks, with significant increases compared to the baseline for two weeks (*P<0.05).

SUMMARY

Analgesic chromaffin-like cells can be generated from an individual's own tissue-derived stem cells by targeted cell reprogramming and these chromaffin-like cells may serve as potential autografts and provide innovative therapies for chronic pain without immunological problems.

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Targeted cell reprogramming produces analgesic chromaffin-like cells from human mesenchymal stem cells

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Running head: Producing analgesic cells for pain management

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ABSTRACT:

Transplantation of allogeneic adrenal chromaffin cells demonstrated the promise of favorable outcomes for pain relief in patients. However, there is a very limited availability of suitable human adrenal gland tissues, genetically well-match donors in particular, to serve as grafts. Xenogeneic materials, such as porcine and bovine adrenal chromaffin cells, present problems, for instance, immune rejection and possible pathogenic contamination. To overcome these challenges, we have tested the novel approach of cell reprogramming to reprogram human bone marrow (BM)-derived mesenchymal stem cells (hMSCs) using cellular extracts of porcine chromaffin cells and we produced a new type of cells, chromaffin-like cells. These chromaffin-like cells generated from the reprogrammed hMSCs displayed a significant increase in expression of human preproenkephalin (hPPE), a precursor for enkephalin opioid peptides, compared to the inherent expression of hPPE in naïve hMSCs. The resultant chromaffin-like cells not only expressed the key molecular markers of adrenal chromaffin cells, such as tyrosine hydroxylase (TH) and methionine enkephalin (Met-enkephalin), but also secreted opioid peptide Met-enkephalin in culture. In addition, intrathecal injection of chromaffin-like cells in rats produced significant analgesic effects without using immunosuppressants. These results suggest that analgesic chromaffin-like cells can be produced from an individual's own tissue-derived stem cells by targeted cell reprogramming and also that these chromaffin-like cells may serve as potential autografts for chronic pain management.

INTRODUCTION:

Transplantation of allogeneic adrenal chromaffin cells provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1,11,13,25). There is often long-term pain relief without analgesic tolerance (one year in humans) (24). Following adrenal chromaffin cell transplantation, application of exogenous opiates further alleviate pain without dosage escalation (25); i.e., there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that chromaffin cell grafts ameliorated the problem of opioid tolerance (12,13). These adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, gamma amino butyric acid, indolalkylamines, and other neuropeptides (15,42). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9,32,33), suggesting that these analgesic effects are mediated by the opioids and catecholamines released by these cells. The anti-tolerance effects produced by the transplantation of adrenal chromaffin cells may be attributed to the synergistic action of the endogenous molecules released by these transplanted cells.

Although transplantation of allogeneic chromaffin cells demonstrates a viable modality of effective treatment for relieving pain and suffering in patients with the promise of ameliorating opioid tolerance, clinical practice has been hindered due to the limited availability of suitable human adrenal gland tissues. Mature chromaffin cells are post-mitotic when they produce enkephalins and catecholamines. Expansion of these cells in culture is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been

extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in rodents and in non-human primates (16,23,32-34,40) with a gradual decline in analgesic efficacy that can be prolonged by administration of immunosuppressants (23). Although chromaffin cells themselves are not very immunogeneic, and highly purified chromaffin cells may minimize immunorejection (4,20), these experimental results suggest that in transplants, xenogeneic chromaffin cells elicit immunological host rejection and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of these transplants. In addition, there has been concern regarding pathogen contamination by these xenogeneic materials, such as bovine spongiform encephalopathy in bovine chromaffin cell transplantation. Thus, the use of xenogeneic chromaffin cells presents serious problems.

The emerging approach of cell extract-based cell reprogramming developed by Håkelien and Collas, et al. (3,5,8) represents a novel technology to furnish the phenotypic characteristics of the target cells to the reprogrammed cells for therapeutic purposes. We previously (41) reported that bone marrow (BM)-derived human mesenchymal stem cells (hMSCs) hold the inherent gene expression of preproenkephalin (PPE), a precursor for enkephalin opioid peptides, such as methionine-(Met-) and leucine-(Leu-) enkephalins, and in culture, are able to release a low basal level of Met-enkephalin, a major neurotransmitter that plays an important role in analgesia by activating opioid receptors (15,33). To address the clinical needs for new and safe alternatives to adrenal chromaffin cells, recently we further reprogrammed these hMSCs with nuclear and cytoplasmic extracts of porcine chromaffin cells and examined the phenotypic development and functional changes of the reprogrammed cells. RT-PCR assays revealed that the expression of human PPE (hPPE) in the

reprogrammed hMSC population was greatly enhanced compared to that of naïve hMSCs (unprogrammed). Immunoblot analysis confirmed that secretion of Met-enkephalin in serum-free culture of reprogrammed hMSCs was significantly augmented compared to secretions released by naïve hMSCs for the same number of cells under identical conditions. Immunocytochemical examination demonstrated strong immunoreactivity in the reprogrammed hMSCs for Met-enkephalin and tyrosine hydroxylase (TH), specific markers for adrenal chromaffin cells. By targeted cell reprogramming, we have successfully developed a new type of cells from the reprogrammed hMSCs, which demonstrates the key phenotypic and functional features of adrenal chromaffin cells and thus termed these cells "chromaffin-like cells" (37,38). Transplantation of these chromaffin-like cells into the spinal intrathecal space of animals produced significant analgesic effects on both Aδ nociceptor- and C nociceptor-mediated responses in a rat thermal pain model. The analgesic effects lasted three weeks without immunosuppression. Preliminary data from some of this work has been reported previously in abstracts (37,38).

MATERIALS AND METHODS

Chromaffin cell isolation, purification and culture: Porcine (1 year old) adrenal glands were obtained from a local slaughterhouse and immediately placed in ice-cold Locke's buffer supplemented with 100 UI/ml penicillin, 100 mg/ml streptomycin, and 0.125 ml/ml fungizone (PSF; Sigma, St Louis, MO) for transport. Preparation of isolation chromaffin cells was carried out using a method reported by us previously (39) with modifications. In brief, adrenal glands were manually perfused three times with warmed (37°C) Locke's buffer followed by digestion of connective tissue with 0.125% collagenase A (Boehringer Mannheim, Mannheim, Germany) in Locke's buffer for 3×5 min at 37°C. At the end of the digestion

period, the chromaffin cells were isolated from the dissected medulla by mechanical dissociation. The harvested cells were filtered through 82 mm nylon mesh and then centrifuged at 1000 rpm/min for 10 min in Locke's buffer. The resulting cells were purified on 39.47% Percoll gradients (Pharmacia Biotech, Uppsala, Sweden) by centrifugation at 12,000 rpm/min for 20 min. The portion of the gradients containing purified chromaffin cells was harvested by aspiration and washed 3 times by centrifugation at 1000 rpm/min in Locke's buffer. Freshly isolated chromaffin cells were suspended and plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) containing Dulbecco's modified eagle medium/F-12 (DMEM/F12, 1:1; Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma, St Louis, MO) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA) fed by replacing culture media twice per week and used for experiments within four weeks after culturing because mature chromaffin cells are post-mitotic and do not survive longer in cultured conditions.

hMSC culture: In total, four samples of hMSCs, which are negative for surface markers associated with hematopoietic cells (e.g., CD11b, CD33, CD34 and CD133 antigens), were obtained from Cambrex (Walkersville, MD). hMSCs were cultured and expanded using the protocol previously developed in our laboratory (30,39,41). In brief, hMSCs were plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) at a concentration of $1x10^5$ cells/cm^2 and cultured in 20 ml growth medium consisting of DMEM (Gibco, Grand Island, NY), an antibiotic-antimycotic mixture (1:100, Invitrogen, Carlsbad, CA), and FBS (Stem Cell Technologies, Vancouver, BC, Canada), incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA), and fed by replacing half of the culture media twice a week. Cells were passaged by incubating with 0.05% trypsin-EDTA (Gibco, Grand

Island, NY) for 5 min at room temperature to gently release the cells from the surface of the culture flask after reaching about 80% confluency. Culture medium was added to stop trypsinization and the cells were centrifuged at 1500 rpm/min for 5 min at room temperature, resuspended, and transferred into new culture flasks at a concentration of $1x10^5$ cells/cm^2 for continuous culture and expansion to reach a sufficient number of cells. hMSCs that underwent less than 10 passages were used for the experiments.

Reprogramming hMSCs with the extracts of porcine chromaffin cells: The technology for cellular extract-based cell reprogramming, originally reported by Håkelien and Collas, et al. (3,5,8), was applied by us with some modifications (37,38).

a. Preparation of porcine chromaffin cell extracts: Cultured porcine chromaffin cells were counted and washed twice in PBS and in cell lysis buffer (20mM HEPES, pH 8.2, 50 mMNaCl, 5mM $MgCl_2$, 1mM dithiothreitol, and protease inhibitors) (Sigma, St Louis, MO), sedimented at 400 ×*g*, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200µl aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed, and confirmed by microscopic observation. The lysate was centrifuged at 15,000 ×*g* for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use. Before applying to cell reprogramming, the protein concentrations of the cell extracts were determined (~30 mg/ml).

b. Permeabilization of hMSCs with SLO: hMSCs were resuspended from the culture, washed in Ca^{2+}- and Mg^{2+}-free PBS, and centrifuged at 120 ×*g* for 5 min at 4°C. The collected hMSCs were resuspended in aliquots of $1x10^5$ hMSCs/100µl of Ca^{2+}- and Mg^{2+}-free PBS in

1.5 ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich, St Louis, MO) at a final concentration of 200 ng/ml and incubated in an H_2O bath at 37°C for 50 min with occasional agitation to mix the cells. The cell samples were then placed on ice, diluted with 200µl cold PBS, and sedimented at 150 ×*g* for 5 min at 4°C.

c. Reprogramming hMSC with the extracts of porcine chromaffin cells: The reversibly permeabilized hMSCs ($1x10^5$) were resuspended in 100µl of the extracts of porcine chromaffin cells in a 1.5ml tube containing an ATP-regenerating system (1mM ATP, 100µM GTP, and 1mM of each NTP, 10mM creatine phosphate, 25µg/ml creatine kinase) (Sigma, St Louis, MO). Cell samples were incubated in an H_2O bath at 37°C for 1 hr with occasional agitation. To reseal the membranes of hMSCs, 1ml of DMEM containing 2mM $CaCl_2$ and antibiotics were added to the tube and incubated at 37°C for an additional 1 hr. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS and reprogrammed hMSCs were transferred to 24-well plate at a concentration of $1x10^4$ cells per well or into 75 cm^2 culture flask at a concentration of $1x10^5$ cells/cm^2 and incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA). Twenty-four hours later, the floating cells were removed. The reprogrammed hMSCs grew well in culture and were expanded continuously, fed by replacing half of the culture media twice per week, and underwent regular cell passages when reached confluence. Cells between passage 2 (1 week after cell reprogramming) and passage 4 (2 weeks after cell reprogramming) were used for experiments and the viability of chromaffin-like cells was examined by typan blue exclusion to be ≥95% at the time point before each experiment.

RT-PCR: The expression of hPPE genes in the population of chromaffin-like cells was analyzed by RT-PCR one week (passage 2) after cell reprogramming. Naïve hMSCs with the

same number of passages served as controls. RNAs from the cells were isolated using TRIzol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and treated with RNAase free DNAase (Promega, Madison, WI). The concentration of RNA was quantified by absorbance at 260 nm. RT-PCR was performed using a SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) by means of specific primer sets for gene hPPE (forward: 5'-ACATCAACTTCCTGGCTTGCGT-3' and reverse: 5'-GCTCACTTCTTCCTCATTATCA-3') and β-actin (forward: 5'-GACAGGATGCAGAAGGAGAT-3' and reverse: 5'-TTGCTGATCCACATCTGCTG-3'). RT-PCR products were quantified using the Qgel 1D program (Stratagene, Cambrige, UK) and expressed as hPPE/β-actin (an internal control) ratio. In total, three independent experiments were performed for this experiment. Statistical analysis was performed using Student's t-tests at a significance of $P<0.05$.

Immunoblot: The production and secretion of Met-enkephalin opioid peptides in chromaffin-like cell culture was examined at one week (passage 2) following cell reprogramming using an established protocol of immunoblot assay as we performed previously (41). Cultured hMSCs with the same number of passages served as controls. In brief, cultured cells were re-suspended from cell culture flasks and transferred onto a 12-well culture plate containing serum-supplemented growth medium at a concentration of $1x10^5$ cells/well. After recovering overnight, the serum-supplemented culture medium was replaced by serum-free culture medium. Twenty-four hours later, the medium was collected and purified with YM-30 microcon (Millipore Corp., Bedford, MA). Purified medium samples (200µl for each) obtained from chromaffin-like cells and naïve hMSCs were applied to Hybond ECL nitrocellulose membranes (Amersham Life Science, Piscataway, NJ) using the Slot Blot Hybridization Manifold (GENEMate, Kaysville, UT). The membranes were blocked with 3%

normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS containing 0.5% Tween 20 (PBST) for 2 hr at room temperature and then incubated with a specific Met-enkephalin rabbit antibody (1:600, ImmunoStar Inc., Hudson, WI) overnight at 4°C. After washing 3 times with PBST, the membranes were incubated with anti-rabbit IgG peroxidase-linked species-specific whole donkey antibody (1:3000, Amersham Biosciences, Piscataway, NJ) for 90 min at room temperature. The membranes were washed with PBS, incubated with an ECL Plus detection reagent for 5 min, and then exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). The films were developed and scanned into a computer. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH). Data was expressed as the mean ± SEM of four independent experiments. Student's t-tests were performed to compare the levels of Met-enkephalin released by chromaffin-like cells with the levels released by naïve hMSCs for the same number of the cells under the same condition of the serum-free cultures. Significance was set at $P<0.05$.

Immunocytochemistry: Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks (passage 4) post-cell reprogramming. Naïve hMSCs served as controls. Cell samples in eight-well culture chambers (Thermo Scientific, Swedesboro, NJ) were washed with PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. To examine the proliferative potential of chromaffin-like cells, cell samples were incubated with 1μM BrdU for 24 hr and then treated with 2N HCL for 30 min. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma, St Louis, MO) and 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min, followed by incubation with sheep anti-BrdU (1:500; Fitzgerald, Concord, MA), mouse anti-TH (1:300; Sigma, St Louis, MO), or rabbit anti-Met-enkephalin

(1:600; ImmunoStar Inc., Hudson, WI) antibodies overnight at 4°C. Then, the cells were washed in PBS and incubated with their corresponding secondary antibodies, including rhodamine (TRITC)-conjugated donkey anti-sheep IgG (1:200; Jackson ImmunoResearch), FITC-conjugated donkey anti-mouse IgG, and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies for 2 hr at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA), and viewed by immunofluorescence microscopy (Zeiss, Jena, Germany). The number of cells fluorescently immunostained by a specific antibody and the number of cell nuclei stained by DAPI were counted in five randomly selected microscopic fields at x200 magnification. In total, three independent experiments were performed and about 50-100 cells were counted for each experiment. The ratio of the cells fluorescently immunostained by specific antibodies to the total number of counted cells was recorded.

Pain behavioral tests: All animal experiments were performed in accordance with federal guidelines for proper animal care and an approved Institutional Animal Care and Use Committee protocol. In total, 12 adult male Sprague Dawley (SD) rats (250-300g body weight) were used. Foot withdrawal latencies were measured before cell transplantation using high (Aδ nociceptor: 6.5°C/second) and low (C nociceptor: 0.9°C/second) rates of radiant heating on the dorsal surface of the feet according to the methods previously reported by us (16,17). The latencies from the onset of the stimulus to foot withdrawal responses were measured over 1 hr at 10 min intervals. Chromaffin-like cells cultured two weeks (passage 4) after cell reprogramming were used. After the baseline for foot withdrawal responses was established, a single dose ($1x10^5$) of chromaffin-like cells in 20μl culture solution was injected intrathecally between the L4-L5 lumbar vertebrae levels into the rats (n=6) under anesthesia with pentobarbital (50mg/kg, i.p.). We have observed previously that

transplantation of this amount of chromaffin-like cells is appropriate to produce optimal analgesic responses in rats (37). Control rats (n=6) received injections of the same amounts of naïve hMSCs. Immunosuppressants were not used for cell transplantation. Foot withdrawal latencies were remeasured 1 week following cell transplantation and then weekly at one week intervals until latencies returned to baseline. Foot withdrawal latencies were expressed as the mean ± SEM. Data from baseline (before cell transplantation) and response latencies at different time points after cell transplantation were compared and analyzed using t-tests followed by Bonferroni post-hoc correction. Significance was set at $p<0.05$.

RESULTS:

hPPE gene expression of chromaffin-like cells: One week after cell reprogramming with the cellular extracts of porcine chromaffin cells, RT-PCR was performed to examine the expression of hPPE. The molecular size of the RT-PCR product for the hPPE gene fragment was 425bp. As expected, naïve hMSCs demonstrated a low level of inherent hPPE gene expression. The chromaffin-like cells generated from the reprogrammed hMSCs showed a significantly enhanced expression profile for gene hPPE compared to that of naïve hMSCs (Fig. 1, $P<0.01$), suggesting that cell reprogramming further increases the expression of hPPE genes in the population of reprogrammed hMSCs.

Met-enkephalin secretion of chromaffin-like cells: In parallel to the time point examined for the expression of hPPE genes by RT-PCR (one week post-cell reprogramming), the serum-supplemented culture medium for chromaffin-like cell and naïve hMSC cultures was replaced by a serum-free culture medium. Twenty-four hours later, the medium was collected and purified for Met-enkephalin detection by immunoblot assays. As shown in Fig. 2, the

level of Met-enkephalin released by chromaffin-like cells was significantly augmented compared to that released by naïve hMSCs for the same number of cells ($1x10^5$ cells/well) in serum-free cultures ($P<0.01$). Although naïve hMSCs were able to produce and release a low level of Met-enkephalin into the serum-free medium, augmented production and secretion of Met-enkephalin opioid peptides in chromaffin-like cells were consistently observed in each of the four independent experiments.

Immunocytochemical examination of chromaffn-like cells: Following cell reprogramming, morphological changes in hMSCs were observed within the first few days; i.e., reprogrammed hMSCs became smaller and rounder. Five days later, the cells reverted to fibroblast-like shapes. These cells expanded in culture at a speed slightly slower (doubling time: about 84 hours) in the first week and recovered at a normal dividing rate (doubling time: about 72 hours) similar to that of naïve hMeSCs. Two weeks after cell reprogramming, immunocytochemical examination showed that most of the resultant chromaffin-like cells (≥90%) expressed a strong immunoreactivity for Met-enkephalin and TH (Fig. 3), specific cytoplasmic markers for adrenal chromaffin cells. Interestingly, BrdU-positive staining was detected in a subpopulation of chromaffin-like cells (data not shown), suggesting that some chromaffin-like cells may have retained a similar proliferative capability as that of hMSCs and could be expandable in cell cultures. These chromaffin-like cells remained stable phenotypes and expanded in cultures with an average cell doubling time of about 72 hours up to one month, undergoing about 8 passages. We did not maintain the culture of the cells post the one month time point after cell reprogramming.

Analgesic effects of chromaffn-like cells: The analgesic effects of the chromaffin-like cells to a noxious thermal stimulus were investigated with the Aδ and C fibers-mediated foot-

withdrawal latency tests in rats. Transplantation of chromaffin-like cells into the subarachnoid space of rats produced remarkable analgesic effects on both Aδ and C fiber-mediated responses, which were evoked by a high and a low heating rate, respectively, and increased foot-withdrawal latencies for three weeks in the absence of immunosuppression, with significant increases compared to the baselines of foot-withdrawal latencies for two weeks (Fig. 4, $P<0.01$). The analgesic effects of chromaffin-like cells were greater for C fiber- than for Aδ fiber-mediated responses. The maximum analgesic effects were observed in the first week following cell transplantation and analgesic efficacy declined gradually with time. Control rats with naïve hMSC transplantation showed similar response latencies to those measured previously in rats without cell transplantation (data not shown). No adverse effect was observed in the rats either with chromaffin-like cell or naïve hMSC transplantation.

DISCUSSION:

Cellular extract-based cell reprogramming technology makes it possible to develop new types of cells by reprogramming one type of cells with extracts derived from a chosen targeted cell type to furnish reprogrammed cells with the phenotypic characteristics of the target cells (3,5,8). For example, incubation of a cell line, 293T fibroblasts, in the nuclear and cytoplasmic extracts of human T-cells resulted in the reprogrammed fibroblast cells taking on T-cell properties, expressing T-cell-specific surface molecules, and assembling the interleukin 2 receptor in response to T-cell receptor CD3 stimulation - a complex regulatory function (8). By using a similar approach, a related study demonstrated the induction of the pancreas-specific genes Pdx1 and insulin in rat primary fibroblasts treated with an extract of rat insulinoma cells (6). In the current studies, we reprogrammed hMSCs with nuclear and cytoplasmic extracts from porcine chromaffin cells and the reprogrammed hMSCs

demonstrated phenotypic and functional characteristics similar to those of true chromaffin cells.

As previous studies have shown, hMSCs are a subset of self-renewing multipotent stem cells and are capable of differentiating into various mesenchymal cell lineages, including bone, cartilage, fat, tendon, and other connective tissues (18,26,28). Several studies have reported that hMSCs can also transdifferentiate into a diverse family of cell types unrelated to their phenotypic embryonic origin, including muscle and heptocytes (14,19,27), as well as neural cells (2,30,35). Recent studies, including ours, have revealed that hMSCs display an inherent gene expression of hPPE and spontaneously release a low level of Met-enkephalin in culture (29,41). These cells are preferred candidates for our targeted cell reprogramming not only because of their latent capability to produce analgesic substances and plasticity for multi-transdifferentiation but also because they are relatively easy to isolate from an individual's own tissues and are able to be expanded in culture with a regular doubling time as well as low levels of senescence during repeated passages. We choose porcine adrenal chromaffin cells as reprogramming materials because these cells share characteristics with human adrenal chromaffin cells in many respects, such as synthesizing and releasing opioid peptides and other pain-inhibitory compounds, including enkephalins and catecholamines (16,40). Also, compared with bovine chromaffin cells, porcine chromaffin cells are more potent in producing analgesia as transplants (16). In addition, porcine chromaffin cells are considered a safe source of cells that are readily available in large quantities - in fact, pigs have been cloned and are now being bred for whole organ transplantation in humans (21,22).

Consistent with previous studies (29,41), our results demonstrate that naïve hMSCs are able to express a low level of hPPE genes and to release a basal level of Met-enkephalins into a

serum-free culture medium. However, chromaffin cell extract-based cell reprogramming significantly increases the expression of hPPE and the production and release of analgesic molecule Met-enkephalin, a neurotransmitter that plays a major role in analgesia by activating peripheral opioid receptors, in reprogrammed hMSCs (Fig. 1 & Fig. 2, $P<0.01$). As shown by our immunocytochemical examination, about 90% of the resultant chromaffin-like cells expressed strong Met-enkephalin and TH (Fig. 3), key markers of chromaffin cells. We did not examine the levels of catecholamines released by chromaffin-like cells in this study, however, TH is an enzyme controlling the rate-limiting step of catecholamine biosynthesis and is specifically found in the cytoplasmic matrix of cells containing catecholamines (31,36), positive immunoreactivity for TH may suggest that these chromaffin-like cells have the potential to produce catecholamines. More interestingly, BrdU immunoreactivity was detected in a subpopulation of chromaffin-like cells (30%), suggesting that these cells may have retained the proliferative properties of hMSCs and may make these cells even more valuable because generation of dividing cells that are expandable in culture would provide a sufficient quantity of such cells for targeted use. In practice, the analgesic potential and secretion activity of chromaffin-like cells for enkephalins can be further manipulated in continuous cultures and thus, become more powerful by using targeted gene transfection and/or cell fusion techniques, as demonstrated in our previous studies (39,41). As revealed by RT-PCR, immunocytochemistry, and immunoblot analyses in our studies, reprogrammed hMSCs demonstrated similar phenotypic and functional characteristics of chromaffin cells by targeted cell reprogramming. Although the mechanism for cell reprogramming is not fully understood, it is suggested that nuclear and cytoplasmic extracts may contain regulatory components that mediate alterations in the gene expression profile of the target genome and promote the nuclear importation of nuclear regulatory components (7,10). It is likely that chromaffin cell

extract-based cell reprogramming switches hMSCs from a mesenchymal program to a chromaffin-like program.

We further investigated the analgesic effects of chromaffin-like cells *in vivo* by spinal transplantation of the cells into the subarachnoid space of the rats. As shown in Fig. 4, transplantation of chromaffin-like cells produced remarkable analgesic effects and significantly increased the foot-withdrawal latencies mediated by both high (Aδ nociceptor) and low (C nociceptor) heating rates (Fig. 4, $P<0.01$). The analgesic effects of chromaffin-like cells lasted for three weeks in rats without immunosuppression. Although we did not make observations on the secretion of chromaffin-like cells past the one week time point of cell reprogramming in our in vitro studies, the analgesic effects of chromaffin-like cells demonstrated in living animals suggest that these cells can continuously release analgesic substances in vivo after transplantation. In addition, a more robust analgesic potential of chromaffin-like cell grafts compared to that of the hPPE-transfected NT2 (human neuron-committed teratocarcinoma) cell grafts as reported in our previous studies was found (17), i.e., a low number of chromaffin-like cells ($1x10^5$) produced similar analgesia effects to that of a high number of hPPE-transfected NT2 cells ($1x10^7$) in the same animal models. The duration of the analgesic effects produced by chromaffin-like cells was similar to that of the porcine chromaffin cells reported previously (16), with a time-dependent gradual decline in analgesic efficacy. Although we did not perform histological examination for the grafted cell fate in this study, these results suggest that host immune responses to the transplanted xenogeneic cells may occur. As demonstrated by our previous study (23), only few xenogeneic cells could be detected in the transplanted animals without immunosuppression, suggesting that administration of immunosuppressants is necessary to maintain long-term survival and long-lasting analgesic effects of xenogeneic cell grafts. Further studies are warranted to determine

the survival rate, phenotypic stability, and secretion activity of chromaffin-like cells *in vivo* by using autologous cell transplantation in related animal pain models.

It is common knowledge that use of immunosuppressants can have severe side effects, including tumor formation, and should be avoided when possible. Transplantation of cells derived from individuals' autologous tissues into the same individuals would be safe and immunocompatible compared to xeno- and allo-transplants. In practice, hMSCs can be derived from a patient's own tissues and could not only be prepared to become isogeneic through this cell reprogramming approach, but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous chromaffin-like cells could be the most desirable alternative to adrenal chromaffin cells for potential therapeutic purposes. Robust and long-lasting analgesic effects of autologous chromaffin-like cells are expected because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the transplanted cells.

FIGURE LEGENDS

Fig. 1. The expression of hPPE gene in the chromaffin-like cells was analyzed by RT-PCR at one week (passage 2) after cell reprogramming. Naïve hMSCs with the same number of passages served as controls. RT-PCR products (425bp) were quantified using the Qgel 1D program (Stratagene, Cambrige, UK) and expressed as hPPE/β-actin (an internal control) ratio. In total, three independent experiments were performed. Statistical analysis showed that hPPE expression of chromaffin-like cells (A) is significantly up-regulated compared to that of naïve hMSCs (B) (*P<0.01, Student's t-test).

Fig. 2. The secretion of Met-enkephalin opioid peptides of chromaffin-like cells ($1x10^5$ cells) in serum-free culture was examined by an Immunoblot at one week (passage 2) after cell reprogramming. Naïve hMSCs ($1x10^5$ cells) with the same number of passages served as controls. Twenty-four hours after culturing, the medium sample was collected and purified for Immunoblot assay. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH). Data was expressed as the mean ± SEM of four independent experiments. Statistical analysis showed that the Met-enkephalin secretion of chromaffin-like cells (A) is significantly augmented compared to that of naïve hMSCs (B) for the same number of cells under the same culture condition (*P<0.01, Student's t-test).

Fig. 3. Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks post-cell reprogramming (passage 4). Naïve hMSCs with the same number

of passages served as controls. Micrographs showed that chromaffin-like cells expressed strong immunoreactivity for tyrosine hydroxylase (a) and Met-enkephalin (c), while naïve hMSCs expressed weak immunoreactivity for tyrosine hydroxylase (b) and Met-enkephalin (d). DAPI stained the nucleus of all cells. Consistent results were received in three independent experiments. Scale bar=20µm.

Figure 4. Effects of intrathecal transplantation of a single dose ($1x10^5$) of chromaffin-like cells on foot withdrawal latencies evoked by low (C nociceptor: 0.9°C/second, squares) and high (Aδ nociceptor: 6.5°C/second, diamonds) heating rates on the dorsal surface of the feet in rats (n=6). Foot withdrawal latencies were measured before cell transplantation (baseline), and remeasured 1 week and then weekly at one week intervals following cell transplantation. Data from baseline and response latencies at different time points after cell transplantation were expressed as the mean ± SEM and compared using t-tests. Follow-up analysis was performed by Bonferroni post-hoc tests. Results showed that transplantation of chromaffin-like cells increased foot withdrawal latencies evoked by both high and low heating rates for at least three weeks, with significant increases compared to baseline for two weeks (*P<0.01, t-tests and Bonferroni correction).

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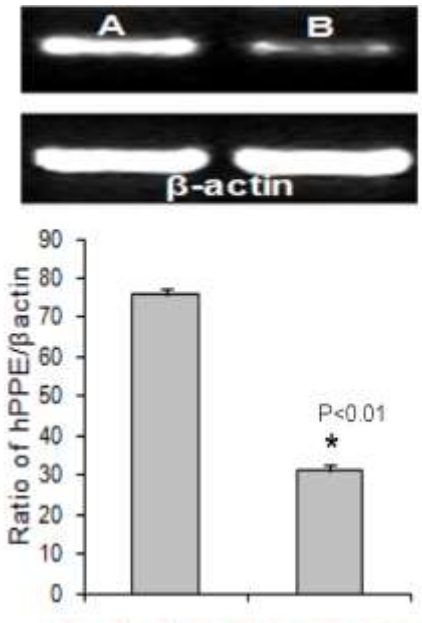

Figure 1

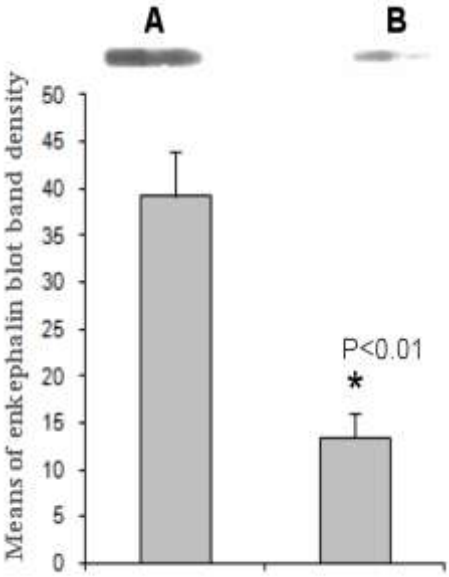

Figure 2

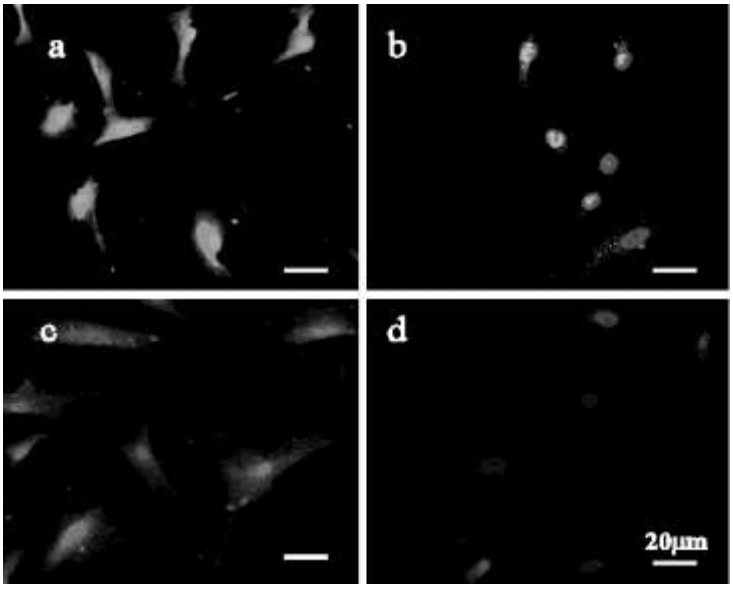

Figure 3

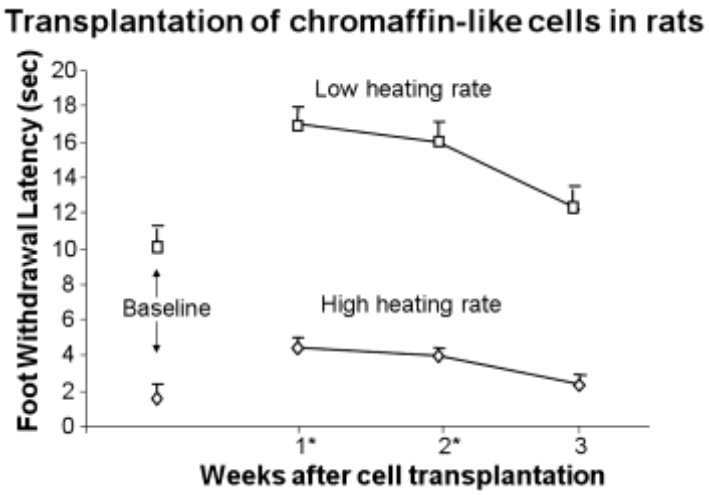

Figure 4